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**MODIFICATIONS OF MYOFILAMENT STRUCTURE AND
FUNCTION DURING GLOBAL MYOCARDIAL ISCHEMIA**

A Thesis Presented

by

MIKE K. WOODWARD

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2016

Department of Molecular and Cellular Biology

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ABSTRACT

MODIFICATIONS OF MYOFILAMENT STRUCTURE AND FUNCTION DURING GLOBAL MYOCARDIAL ISCHEMIA

SEPTEMBER 2016

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Cardiac arrest is a prevalent condition with a poor prognosis, attributable in part to persistent myocardial dysfunction following resuscitation. The molecular basis of this dysfunction remains unclear. We induced cardiac arrest in a porcine model of acute sudden death and assessed the impact of ischemia and reperfusion on the molecular function of isolated cardiac contractile proteins. Cardiac arrest was electrically induced, left untreated for 12 min, and followed by a resuscitation protocol. With successful resuscitations, the heart was reperfused for 2 h (IR2) and the muscle harvested. In failed resuscitations, tissue samples were taken following the failed efforts (IDNR). Actin filament velocity, using myosin isolated from IR2 or IDNR cardiac tissue, was nearly identical to myosin from the control tissue in a motility assay. However, both maximal velocity (25% faster than control) and Ca^{2+} sensitivity (pCa_{50} 6.57 ± 0.04 IDNR vs. 6.34 ± 0.07 control) were significantly ($p < 0.05$) enhanced using native thin filaments (actin, troponin, and tropomyosin) from IDNR samples, suggesting that the enhanced velocity is mediated through an alteration in muscle regulatory proteins (troponin and tropomyosin). Mass spectrometry analysis showed that only samples from the IR2 had an increase in total phosphorylation levels of troponin (Tn) and tropomyosin (Tm), but both IR2 and IDNR samples demonstrated a significant shift from mono-phosphorylated to bis-

phosphorylated forms of the inhibitory subunit of Tn (TnI) compared to control. This suggests that the shift to bis-phosphorylation of TnI is associated with the enhanced function in IDNR, but this effect may be attenuated when phosphorylation of Tm is increased in tandem, as was observed for IR2. There are likely many other molecular changes induced following cardiac arrest, but to our knowledge, these data provide the first evidence that this form cardiac arrest can alter the in vitro function of the cardiac contractile proteins.

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LIST OF ABBREVIATIONS

IDNR	non-successful resuscitation after 20 min
IR2	successful resuscitation followed by 2 h of reperfusion
ROSC	return of spontaneous circulation
Tn	troponin
TnI	inhibitory subunit
TnC	subunit that binds calcium
TnT	subunit that binds tropomyosin
Tm	tropomyosin
NTF	native thin filament
HIC	hydrophobic interaction chromatography

CHAPTER I

INTRODUCTION

Research must be conducted to discover a molecular basis for the myocardial dysfunction that occurs due to global ischemia. Sudden cardiac arrest is typically precipitated by ventricular fibrillation or pulseless ventricular tachycardia, a life-threatening condition characterized by an abrupt loss of blood flow resulting in progressive global ischemia [Neumar et al., 2008]. The earliest biochemical changes that occur in the myocytes during an ischemic event are the accumulation of metabolites including: hydrogen ions (H^+), inorganic phosphate (P_i), and adenosine diphosphate (ADP) [Bolli and Marban, 1999 , Allen and Orchard, 1987 , Schaefer et al., 1990 , Elliot et al., 1992]. These molecules exert direct depressive effects on the mechanical function of the contractile apparatus, however levels of these molecules quickly (< 5 minutes) return to normal with return of blood flow and therefore cannot explain the persistent decrease in cardiac function [Bolli and Marban, 1999 , Allen and Orchard, 1987 , Schaefer et al., 1990 , Elliot et al., 1992]. At a molecular level, force and power in the heart result from the calcium (Ca^{2+}) dependent sliding of actin-based thin filaments past myosin-thick filaments in a process driven by the hydrolysis of adenosine triphosphate (ATP). Thus, the more likely cause of post-arrest myocardial dysfunction are the persistent structural modifications of the contractile apparatus similar to those which occur in models of localized ischemia [Rao et al., 2007]. Several forms of changes have been seen including but not limited to: the contractile proteins incurring oxidative damage [Tiago et al., 2006 , Alvarez and Radi., 2003 , Dean et al., 1997], post-translational modifications [Rao et al., 2007 , Han and Ogut, 2010 , Han and Ogut,

2011 , Christopher et al., 2009], or degradation by proteases [Dean et al., 1997 , Sawicki et al., 2005 , Day et al., 2007] as a result of ischemia.

Successful resuscitation with return of spontaneous circulation (ROSC) can restore blood flow to near normal levels within minutes of an event, however the prognosis for revived patients remains quite poor with only ~10 % surviving to hospital discharge if it occurs outside of a hospital [Eisenberg and Mengert, 2001 , Schoenenberger et al., 1994] and only improves to 23% if it occurs in a hospital setting [Roger et al., 2012]. After resuscitation many patients experience “postcardiac arrest syndrome,” characterized by a persistent mechanical myocardial dysfunction in the absence of large morphological changes [Kern, 2002], which is believed to be a leading cause of the high morbidity and mortality following cardiac arrest [Neumar et al., 2008]. The most notable structural changes that have been observed include post-translational modifications to myosin and troponin subunit I (TnI), such as cleavage, oxidative damage, or phosphorylation, which may have resulted in the marked decrease in contractile function [Monasky et al., 2011 , Rao et al., 2007].

In both research and clinical settings the serum levels of TnT (the subunit that interacts with tropomyosin (Tm)) and TnI can also be measured to determine the severity of myocardial damage and necrosis that caused the release of Tn into the serum [Scheitz et al., 2011 , Park et al., 2011 , Lotze et al., 2011]. However the molecular basis of the persistent mechanical dysfunction are unclear and thus the extremely poor prognosis is unclear [Chalkias and Xanthos, 2012 , Bolli and Marban, 1999]. For example it is unknown if cardiac arrest directly affects the function of contractile proteins that give rise to the force and power generating capacity of the heart [Chalkias and Xanthos, 2012].

This induced global ischemia closely mirrors a clinical setting where human patients whose heart contractions have ceased and have been revived before arriving at the hospital. These patients still have decreased contractile function post resuscitation/reperfusion and high mortality rates. Therefore this work could have important implications for understanding the molecular basis for this highly prevalent condition. These types of investigations could provide crucial insight for the development of novel treatments for this deadly condition.

Specific Aims

The goal of this study was to determine the effect of ischemia and ischemia-reperfusion injury on the function of contractile proteins that help explain the long term depression in cardiac function following these types of events. A more effective understanding of the molecular basis for the depressed cardiac function could potentially lead to improvements in understanding how to improve human heart health. We hypothesized that the isolated cardiac myosin from post-ischemia-reperfusion animals would have a decreased ability to translocate actin filaments and that this would be in part attributable to effects on myosin. Also native thin filaments (NTF), composed of actin and the regulatory proteins Tn and Tm, were isolated and examined since previous studies implicated regulatory protein damage (cleavage and post-translational modifications). We also hypothesized that if there were any alterations (cleavage or phosphorylation) to these regulatory proteins, then it would result in an altered response to Ca^{2+} and would likely result in decreased contractility.

To accomplish these goals we isolated the myosin and NTF from cardiac tissue samples and used the *in vitro* motility assay to determine if there was any change in

function compared to control (hearts that did not undergo ischemia and/or reperfusion). The *in vitro* motility assay allowed us to view actin filaments and NTF as they translocated over a bed of isolated cardiac myosin and flow-cell conditions were altered to examine their impact. This allowed us to analyze the velocity, determine a change in contractile function, and infer a mechanistic change that could have resulted in the altered function. Also specific gel electrophoresis techniques were attempted to determine if there were changes in the phosphorylation status of certain proteins or if cleavage had occurred. These results were inconclusive and so we employed electrospray ionization liquid chromatography tandem mass spectrometry to gain much more accurate data on changes in phosphorylation and cleavage.

CHAPTER II

LITERATURE REVIEW

Calcium and Actomyosin Interaction

Cardiac muscle is able to produce force because of the cyclical interaction of actin and myosin, the two key contractile proteins. Myosin is a molecular motor which can bind to actin and hydrolyze ATP to produce mechanical force or generate motion. Figure 1 displays how the binding of ATP, binding of actin, and hydrolysis follow a cyclic interaction allowing for myosin to bind, produce force, and release from actin to reinitiate the cycle. The displacement caused by myosin's power-stroke thus causes the thick and thin filament to slide over each other, which causes the contraction of muscle tissue.

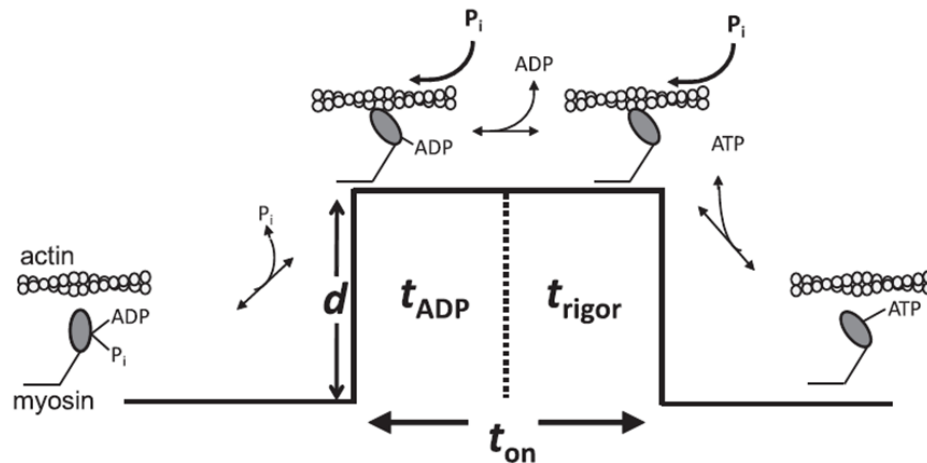


Figure 1. The Crossbridge Cycle. A schematic representation of the crossbridge cycle with d as the displacement and t_{on} as the duration that the myosin is strongly bound to actin, which is composed of t_{ADP} and t_{rigor} . The duration of time the cycle when ADP bound myosin is bound to actin is t_{ADP} . t_{rigor} is the time at which actin and myosin are

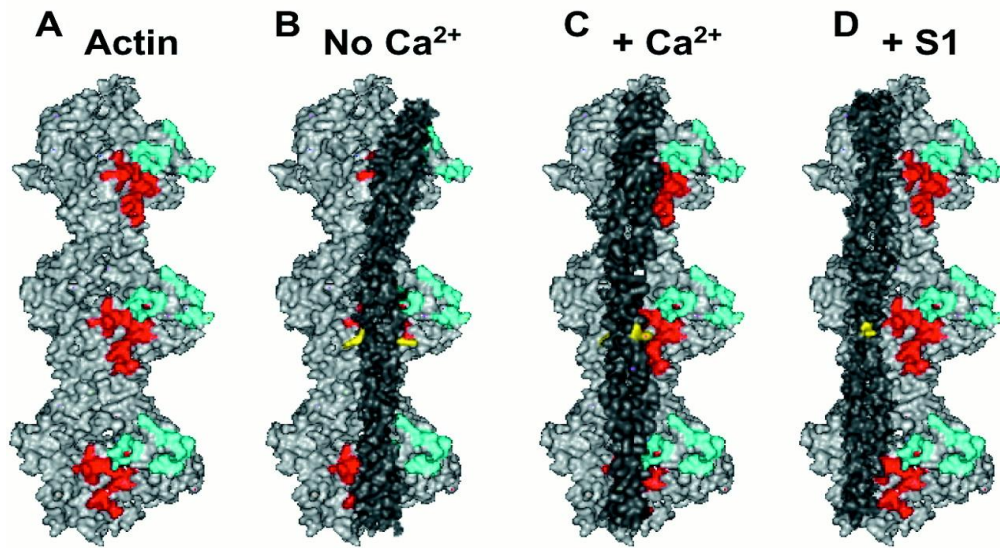


Figure 3. Tropomyosin and the actin filament. Skeletal muscle thin filament: A) only actin, B) with no Ca²⁺, C) with Ca²⁺, and D) decorated with myosin S1. The light green areas are weak myosin binding sites, the red areas are strong myosin binding sites, and the dark filament parallel to the gray actin strand is the Tm. Originally from [Gordon et al., 2001].

In Figure 3B there is no Ca²⁺ able to bind Tn and thus Tm remains and prevents access to the myosin binding sites, but with a high Ca²⁺ state [Fig 3C] Tm moves away from actin's myosin binding sites. Decoration with myosin S1 [Fig 3D] however, demonstrates that with the binding of myosin S1 heads that there is an even greater movement of Tm farther away from the myosin binding sites. This implies a 3 state model that suggests Tm position is modulated by Tn, Ca²⁺ binding, as well as myosin binding. A blocked state in which there is low [Ca²⁺] and myosin cannot bind, but upon Ca²⁺ binding TnC the Tm is no longer strongly held blocking myosin binding sites (called

the closed state) and so a myosin head is able to bind. Once in the closed state and a myosin head is able to bind, it prevents the Tm from blocking adjacent sites, which allows more myosin to bind and propagate this open state across the filament. Proper Ca^{2+} dependent regulation is necessary for normal contraction of both skeletal and cardiac muscle. This is especially crucial in the heart where efficient pumping of the blood requires the careful coordination of systole (ventricular contraction/ejection) and diastole (relaxation/filling).

During acute myocardial ischemia there are a host of intracellular changes including the accumulation of H^+ that affect the contractile process. Myocardial ischemia is a partial or complete reduction of blood flow to cardiac muscle tissues, which deprives the muscle cells of the substrates necessary for energy production but also causes the build-up of cellular metabolites [Allen and Orchard, 1987]. During ischemia, ATP production through oxidative phosphorylation stops, causing a build-up of phosphate products (metabolites), which can lead to arrhythmias and a decrease in tension production [Allen and Orchard, 1987]. This can only be remedied if there is an increase of ATP generation by the re-initiation of oxidative phosphorylation or a decrease in ATP usage, although it is the H^+ and Pi , not the reduced ATP that causes reduced contractility [Allen and Orchard, 1987].

Myocardial ischemia for more than one hour can lead to permanent damage, but reperfusion, the restoration of blood flow to the tissues, can actually accelerate the damage [Allen and Orchard, 1987]. Reperfusion is not a perfect solution to ischemia, although if the tissue is reperfused before or during the early ischemic contracture then normal pressure is completely restored [Allen and Orchard, 1987]. However, if the tissue

is reperfused long after the ischemic contracture has occurred, then the pressure does not recover and can lead to cell damage advancement and the subsequent release of intracellular contents [Allen and Orchard, 1987]. This is deemed reperfusion damage, which occurs because Ca^{2+} is returned to the tissues after a period with low Ca^{2+} levels and this sudden accumulation can cause cellular damage.

There are several theories as to the etiology of the reduced contractility from an ischemic attack, which range from issues with action potential generation, incorrect Ca^{2+} delivery to the contractile proteins, or some alteration and damage to the proteins themselves [Allen and Orchard, 1987]. Each is likely partially responsible for the reduced function. A covalent modification of Tn such as phosphorylation could lead to a loss of Ca^{2+} affinity or a competition between Ca^{2+} and other cations [Allen and Orchard, 1987]. Porcine and murine (rats and mice) models of injury incurred from ischemia and reperfusion have displayed possible proteolytic cleavages (especially of TnI), phosphorylation of myosin and the regulatory proteins, and modifications due to reactive oxygen species [Rao et al., 2007].

Changes in Calcium Sensitivity and Tension

Global ischemia occurs after sudden cardiac arrest, which causes a halt in myocardial contractions and thus blood circulation ceases as well. During this ischemic episode, there is reduced supply of necessary nutrients and an accumulation of harmful metabolites. The changes that occur can be severe and alter the myocardium's ability to properly respond to Ca^{2+} [Fig 4]. In Figure 4C, the tracings were obtained from reperfused cardiac tissue that has undergone ischemia there is an increase in intracellular $[\text{Ca}^{2+}]$. Figure 4D shows that force is reduced due a loss of Ca^{2+} sensitivity, which can be

seen as a rightwards shift in the force-pCa curve. Although there are differences between acidosis [Fig 4A , Fig 4B] where there is no difference in intracellular Ca^{2+} and yet there is still a large loss in Ca^{2+} sensitivity and a reduction in force. Also in a failing heart [Fig 4E , Fig 4F] there is actually an increase in Ca^{2+} sensitivity, which results in a leftwards shift in the pCa curve. Not all conditions have the same impact on Ca^{2+} sensitivity, but each pathology causes detrimental effects on force production and alterations of the response to Ca^{2+} . As cardiac contractions are precisely controlled events, any alteration to cardiac Ca^{2+} sensitivity could severely affect its ability to produce force.

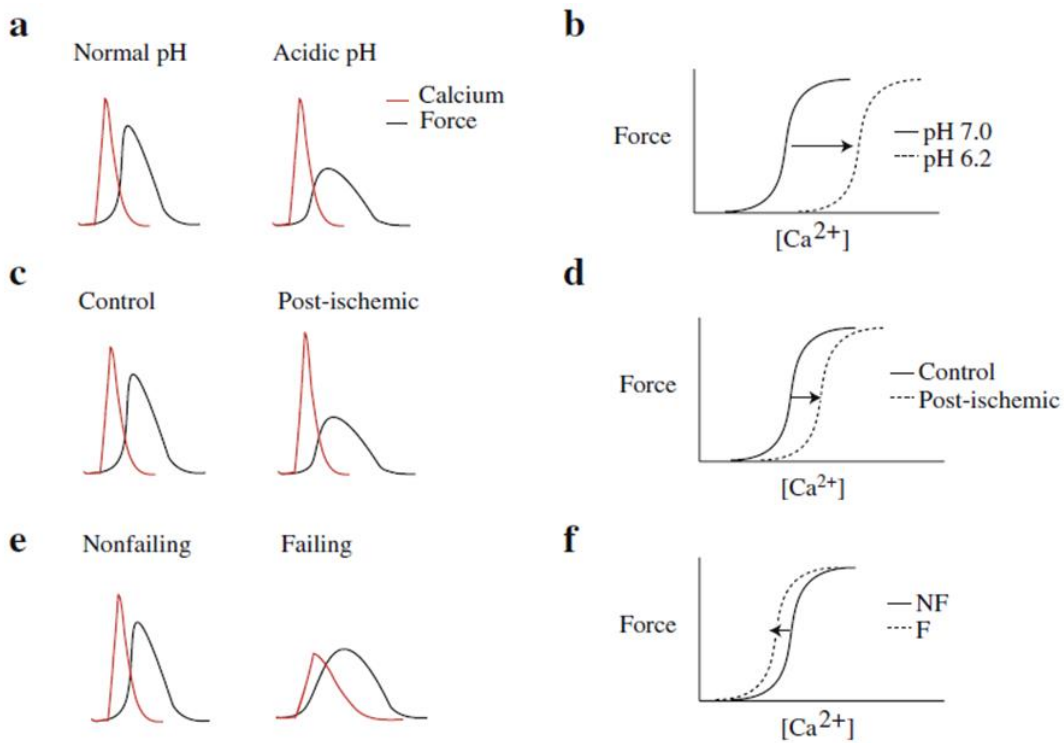


Figure 4. Changes in force- $[\text{Ca}^{2+}]$ relationships. A, C, and E are tracings of the force and cytosolic $[\text{Ca}^{2+}]$ in different physiological environments. B, D, and F are the pCa-force relationships. The effect of acidosis is shown in A and B, the effect of ischemia is

shown in C and D, and the effect of a failing heart is shown in E and F. Originally from [Day et al., 2007].

One research study applied a low flow ischemia model on porcine hearts, specifically fibers from the left ventricle supplied by the left anterior descending coronary artery, which can more effectively simulate ischemia in humans with coronary heart disease than other animal models. In this study the term “stunned” was used to refer to post-ischemic myocardium that had depressed contractile function, which could reverse over a few hours or days. This decreased function was hypothesized to occur because of decreased Ca^{2+} sensitivity and so the researchers directly tested if the myofilaments had reduced post ischemia Ca^{2+} sensitivity. Using an open-chest *in vivo* porcine heart model they obtained biopsies of myocardium before and after ischemia was induced and then subsequently permeabilized the fibers in order to measure Ca^{2+} and tension levels. This *in vivo* study allowed them to look at subsequent measurements of Ca^{2+} sensitivity and tension at different areas of the left ventricle and over the time span of the whole experiment. It was discovered that maximal Ca^{2+} activated tension generating ability after ischemia ($2.77 \pm 0.51 \cdot 10^4 \text{ N/m}^2$) did not differ much from the control ($2.69 \pm 0.27 \cdot 10^4 \text{ N/m}^2$) [Hofmann et al., 1993]. Also ischemia did appear to have induced a significant Ca^{2+} sensitivity reduction as the pCa required for half-maximal activation of tension decreased from 5.88 ± 0.05 control to 5.69 ± 0.03 post-ischemia [Hofmann et al., 1993]. This decreased sensitivity likely caused reduced velocity and reduced tension generating ability at low Ca^{2+} concentration levels.

Oxidative Damage

There has also been evidence of an increase in nitric oxide, superoxide, peroxynitrite (ONOO⁻), and hydroxyl radicals that can cause damage to cells and especially to the muscle proteins themselves [Rao et al., 2007]. Some of the damage can be prevented by reducing the toxicity of the radicals or removing the damaged proteins, although the cell cannot always degrade the damaged proteins and thus their altered or loss of function remains part of the system [Dean et al., 1997]. When the proteins are oxidized it often leads to a loss of function or unfolding, which in turn can allow for more accessibility to proteases [Dean et al., 1997]. Reactive oxygen species (ROS) can damage macromolecules in many cell types and play key roles in many diseases such as ischemia. During ischemia there can be an increase in the levels of these damaging compounds. One such compound called (ONOO⁻) has been shown to cause protein modifications and has reduced maximum isometric tension at high concentrations [Alvarez and Radi, 2003]. It is formed when nitric oxide and superoxide radicals react and this ONOO⁻ product can permeate biological membranes and act as both a nitrating agent and an oxidizer [Alvarez and Radi, 2003]. ONOO⁻ can directly interact with amino acid residues and even produce more free radicals that can continue to cause more protein alterations. Primarily residues containing sulfur (Cys, Met) or aromatic groups (Trp, Tyr, Phe, His) are the first to react with these free radicals [Alvarez and Radi, 2003]. Another study showed that there were velocity reductions for myosin treated with ONOO⁻ and that this may cause modifications to myosin binding protein-C instead of myosin itself at 10uM [ONOO⁻] [Snook et al., 2007]. There were also some functional losses due to actin nitration as well, although bare actin suffered a larger dysfunction compared to

regulated actin at 10uM and 100uM [ONOO-] [Snook et al., 2007]. This may have been due to Tm and Tn subunits interfering with ONOO- access to key sites, which was supported from data displaying that the two proteins showed no nitration at 10uM and only a slight amount at 100 uM [Snook et al., 2007].

Other damaging compounds such as superoxide and nitric oxide could increase in prevalence and lead to damage during myocardial ischemia and reperfusion in a murine model [Liu et al., 1997]. It was discovered that superoxide in tissues of the ischemic cardiac muscle region were increased by 140%, and nitric oxide levels increased by 90%, and nitric oxide synthase activity was increase by 212%, while the non-ischemic regions were unchanged [Liu et al., 1997]. This data displayed that nitric oxide and superoxide formation were increased after reperfusion of ischemic heart tissue and that they may be implicated in reperfusion injury.

Nitric oxide production increases during early reperfusion and can react with superoxide to form the damaging ONOO-, which can lead to protein alterations and a reduction in function. It has also been seen that adding superoxide dismutase or halting nitric oxide synthase activity eliminated the nitric oxide increase [Zweier et al., 2001]. Also there was an over two fold recovery of contractile function in hearts treated with inhibitors of superoxide dismutase or nitric oxide synthase, which displays possible techniques to reduce these oxidative species [Zweier et al., 2001]. After myocardial infarction and heart failure nitric oxide synthase is expressed and the more nitric oxide produced lead to more cardiac dysfunction [Feng et al., 2001]. After myocardial infarction in mutant mice with knockout nitric oxide synthase expression there were a lower mortality rate and improved contractility [Feng et al., 2001]. Nitric oxide produced

by endothelial nitric oxide synthase-3 however has been shown to limit the damage from ischemia-reperfusion and it has been shown that overexpression of this gene specifically in cardiomyocytes improved cardiac function [Elrod et al., 2006]

Free radicals can damage cellular macromolecules by highly reactive chain reactions that involve reactive compounds removing electrons from other molecules and thus creating more reactive substances [Dean et al., 1997]. In reperfused rabbit hearts there were increases of semiquinone, reactive oxygen species, a nitrogenated radical, and hydroxyl radicals that can all be generated in post-ischemic cardiac muscle and could cause damage [Zweier et al., 1989]. Superoxide dismutase, deferoxamine, and mannitol were able to reduce the increase of the reactive oxygen species and thus could also be reasonable strategies to decrease the oxidative damage that can occur [Zweier et al., 1989]. After ischemia the subsequent reperfusion causes a large release of free oxygen radicals and expression or addition of superoxide dismutase immediately before reperfusion reduces the levels to closer to non-reperfused [Grill et al., 1992]. Another avenue of protection against ischemia-reperfusion damage is the MCP-1 (monocyte chemoattractant protein-1), which was expressed in transgenic mice after global ischemia-reperfusion and lead to protection against reactive oxygen species induced cardiac dysfunction [Morimoto et al., 2008]. *In vitro* experiments showed that ischemia-reperfusion caused an increase in MCP-1 mRNA expression [Morimoto et al., 2008]. Mice with cardiomyocyte specific MCP-1 overexpression stopped superoxide formation during ischemia-reperfusion and there was higher superoxide dismutase activity in the transgenic mice than wild type [Morimoto et al., 2008]. These oxidative species can

cause damage such as nitrosylations, which can be detected by specific gels (S-nitrosylated protein detection kit, Cayman Chemical Company, Ann Arbor, Michigan).

Possible Myosin Alterations

It is known that phosphorylation can play a role in regulating myosin activity, which leads to reason that changes in phosphorylation can alter contractile function [Alamo et al., 2008]. The phosphorylation of the regulatory light chains can interact within the same S1 head or between the 2 myosin heads as well [Alamo et al., 2008]. This phosphorylation of the regulatory light chain may reduce the interactions between the 2 myosin heads, thus allowing myosin to interact with actin and produce contractile force [Alamo et al., 2008].

One study discovered that in human heart failure due to mitral regurgitation that there was no change to the actomyosin ATPase activity, contrary to evidence found in some animal studies [Nguyen et al., 1996]. Tissue samples were obtained from the anterior segment of the left ventricular subepicardial wall of the hearts and the isolated myosin was analyzed with *in vitro* motility assays [Nguyen et al., 1996]. The mean velocity of the normal left ventricle function tissue was 1.3 ± 0.3 $\mu\text{m/s}$ and in the mitral regurgitation heart failure it was 1.0 ± 0.3 $\mu\text{m/s}$, which was not a significant difference [Nguyen et al., 1996]. One can infer that the myosin did not have mechanical or ATPase activity changes from mitral regurgitation heart failure in terms the motion producing capacity, but this does not prove that there were no physical modifications [Nguyen et al., 1996].

If cardiomyocytes are pharmacologically preconditioned (with adenosine) *in vivo* to protect against further ischemic events there is an increase in phosphorylation of 2 sites

to myosin light chain 1 (MLC-1) [Arrell et al., 2001]. This suggests the phosphorylation of MLC-1 could be part of the molecular changes of preconditioning that can prevent some of the damage from ischemia. Although there is evidence that ischemia itself does not cause a change in phosphorylation. It has been displayed that in perfused heart fibers there was $86.6 \pm 2.5\%$ unphosphorylated MLC-1 and ischemic fibers with $86.5 \pm 2.9\%$ unphosphorylated MLC-1 [Han and Ogut, 2010]. As well as $76.3 \pm 2.8\%$ unphosphorylated Myosin Light Chain-2 (MLC-2) in perfused fibers and $73.1 \pm 2.7\%$ unphosphorylated MLC-2 in ischemic fibers [Han and Ogut, 2010]. Their data showed no significant changes in light chain phosphorylation status in perfused and ischemic rat cardiac muscle fibers, which helped focus attention on the other sections of myosin that may actually be modified.

Areas of mouse heart that were not part of the myocardial infarction site could have protein modifications as well. These remote areas are part of the overall left ventricular dysfunction, or decreased contractile function, brought on by localized myocardial infarction. One study found the function of infarcted heart myosin was reduced $0.56 \pm 0.11\%$ (adjacent) and $0.60 \pm 0.16\%$ (remote) of the control [Rao et al., 2007]. There was also only 85% of control velocity (control was 4.9 ± 0.2 $\mu\text{m/s}$) in the adjacent zone for moving filaments *in vitro* as well as a decrease in the number of actin filaments bound to the motility surface, which was likely due to impaired actin-myosin binding [Rao et al., 2007]. This study isolated identical myosin concentrations (250 $\mu\text{g/mL}$) from each muscle tissue area sampled to ensure that the number of myosin on the *in vitro* surface was not the reason for the decreased function and velocities found in both the adjacent and remote areas [Rao et al., 2007].

Myosin was analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and stained with 5-IAF (a fluorescent dye) showed that there was a $25 \pm 1\%$ decrease in reactive cysteine-sulfhydryls in the remote zone compared to the control, which meant that some available reactive cysteines were oxidized [Rao et al., 2007]. Alterations to one of these reactive cysteines could possibly have led to unfolding of the protein and thus a loss of function. Also SDS-PAGE showed no cardiac myosin isoform changes [Rao et al., 2007]. This experiment allowed one to theorize that left ventricular dysfunction in the remote area after a myocardial infarction may be due to some type of modification to myosin. The myosin dysfunction may have been caused by oxidative stresses that lead to modifications of the heavy chain or myosin binding protein-C [Rao et al., 2007]. Another study displayed that ONOO⁻ inhibited the S1 actin activated ATPase rate up to 50% and that ONOO⁻ likely oxidized the reactive cysteines (C707 and C697) on Myosin S1 fragment, which could also cause the protein to partially unfold and thus increase access for protease degradation [Tiago et al., 2006]. This degradation could be due to the activity of matrix metalloproteinase-2, which normally acts outside the cell, but has been shown to cleave MLC-1 and result in altered cardiac function *in vitro* [Sawicki et al., 2005]. Inhibiting matrix metalloproteinase-2 reduced cleavage of proteins from IR (specifically MLC-1 was identified by mass spectrometry). MLC-1 can be another target for matrix metalloproteinase-2 and this cleavage may lead to the reduced cardiac function post IR [Sawicki et al., 2005].

Possible Troponin Alterations

Alterations to Tn are one of the most likely reasons for the decreased contractile function post-ischemia. In an *in vivo* permeabilized fiber study of rat cardiac muscle,

specifically the anterolateral papillary, it has been shown that there are changes in phosphorylation of two subunits of Tn (TnI and TnT) [Han and Ogut, 2010]. A ligature was secured on one of the left coronary arteries, which restricted the blood flow for 30 minutes (ischemia), and then it was removed to allow reperfusion for 60 minutes. Ischemia and reperfusion treatment resulted in reduced force and Ca^{2+} sensitivity accompanying a loss of maximal force, an increased Ca^{2+} EC50 (Ca^{2+} concentration needed to achieve the half maximal force), and a clear reduction in stiffness [Han and Ogut, 2010]. A 2-dimensional SDS-PAGE was used to determine if TnT was either mono or bis-phosphorylated. It was discovered that perfused cardiac tissue contained $22.1 \pm 2.2\%$ mono-phosphorylated and $77.6 \pm 2.3\%$ bis-phosphorylated TnT [Han and Ogut, 2010]. In ischemic fibers there was an increase of mono-phosphorylated-TnT to $39.6 \pm 3.0\%$ and a decrease of bis-phosphorylated-TnT to $60.3 \pm 2.9\%$ [Han and Ogut, 2010]. Also the phosphorylation of Ser23/24 on TnI of perfused cardiac muscle was discovered to be $100 \pm 7.4\%$ and this decreased to $65.9 \pm 16.2\%$ in ischemic fibers [Han and Ogut, 2010]. This suggests that myosin is unlikely to be the cause for decreased cardiac muscle function, but the thin filament regulation is very likely to have several modifications that can result in altered function [Han and Ogut, 2010]. The reduced initial attachment rate found in the ischemic tissue displayed that the Ca^{2+} controlled thin filament activation was likely the causal element [Han and Ogut, 2010]. The TnT dephosphorylation could somehow influence the status of the thin filament and thus alter the transition of crossbridges to and from attachment states. This data clearly implicates TnT and TnI modification in controlling contractile capabilities.

Isolated Tn from end-stage failing and non-failing human hearts have also been used in the regulated *in vitro* motility assay and functional changes occurred. Tn from non-failing cardiac tissue at pCa5.4 displayed increases in thin filament (actin-Tn) velocity after addition of Tn from both failing- and non-failing human hearts, however the magnitude of velocity change was significantly larger with the non-failing heart Tn ($52 \pm 4\%$) than the failing-heart Tn ($35 \pm 2\%$) [Knott et al., 2002]. There was an increased sensitivity to Ca^{2+} for the failing heart Tn as the EC50 for failing heart decreased 1.76 ± 0.20 fold as compared to non-failing cardiac Tn and filament velocity was also found to have decreased 1.89 ± 0.62 fold in the failing heart tissue [Knott et al., 2002]. This data demonstrated that a functional alteration to Tn could affect Ca^{2+} sensitivity and contractile function of cardiac myofibrils.

In another study, low blood flow ischemia was performed for 3 days to the left anterior descending artery in a murine model. Using phospho-specific antibodies it was discovered that there may have been Ser23/24 and Ser43/45 phosphorylation changes on TnI [Christopher et al., 2009]. This reduction of Ser23/24 phosphorylation on TnI had been seen in other studies as well [Han and Ogut, 2010]. Additionally affinity chromatography displayed that TnI had reduced affinity for TnC, which increased the likelihood that modifications of TnI can contribute to reduced contractile function [Christopher et al., 2009].

There are 2 isoforms of TnI in humans and there is a transition from ssTnI (slow skeletal) expressed only in embryonic and neonatal hearts and cTnI (cardiac), which replaces the embryonic isoform over time [Day et al., 2007]. They are 70% homologous, but cTnI has a 32 amino acid sequence that can be targeted for phosphorylation by cAMP

dependent protein kinase A and this phosphorylation site can lead to decreased Ca^{2+} sensitivity and cardiac myocyte relaxation [Day et al., 2007]. Also there has been a lot of research displaying that TnI is cleaved by Ca^{2+} activated calpain (a proteolytic enzyme) and can result in the decreased myofilament function [Day et al., 2007].

In addition, it has been shown that in an expressed truncated 1-192 cTnI mutant there is an approximately 9° shift towards the inner groove of the actin filament when activated with Ca^{2+} alone [Galinska et al., 2010]. This shift in equilibrium (28 to 57% enhanced C state) towards the open state would suggest that upon cleavage of the last 17 residues of cTnI there is an “enhanced C-state” that can increase the chance for myosin to bind and thus allow for earlier activation of the filament (less Ca^{2+} needed i.e. increased Ca^{2+} sensitivity) [Galinska et al., 2010]. Although this suggests that there is only a difference when Ca^{2+} is bound as the frequency of the Tm occurring in the blocked state remained virtually identical.

Possible Tropomyosin Alterations

Tm can be phosphorylated, which can affect the way the thin filament is regulated and thus affect contractility. There can be a significant change in the phosphorylation level in muscle tissue isolated from the area completely remote from a site of localized infarction and the phosphorylation of Tm may be caused by oxidative stress from the ischemic area [Rao et al., 2007]. Another experiment, which involved rabbit fast skeletal leg muscles, displayed phosphorylation of alpha-alpha-Tropomyosin ($\alpha\alpha$ -Tm) and TnT [Heeley, 1994]. The five parts of striated muscle thin filaments (actin, Tm, TnT, TnC, TnI) allow for Ca^{2+} controlled contraction activity and TnT actually binds to Tm at two different sites. Different combinations of isomorphs of Tm and TnT can have different

Ca²⁺ dependent tension levels [Heeley, 1994]. The phosphorylation of Ser283 at site one of Tm and phosphorylation of acetyl-Ser 1 of TnT can change Ca²⁺ related tension and so the purpose of the study was to display the effects of *in vivo* modification on thin filament function [Heeley, 1994]. Using fully phosphorylated $\alpha\alpha$ -Tm, TnT, and T1 (T1 is a fragment of TnT), it was discovered that phosphorylated Tm increased the activating properties of the regulatory thin filaments [Heeley, 1994].

Fragment T1 had equal affinity to $\alpha\alpha$ -Tm whether the Tm was phosphorylated or not, but the TnT and phosphorylated $\alpha\alpha$ -Tm affinity was stronger than the control [Heeley, 1994]. With non-phosphorylated $\alpha\alpha$ -Tm, T1 down regulated actin-activated myosin-S1 ATPase activity and this displayed that T1 of the TnT protein has modulating effects on this ATPase activity, although TnI bound to Tm had a more significant reduction in ATPase activity and thus was likely a more important factor [Heeley, 1994]. Activation of myosin-S1 was twice as fast for phosphorylated $\alpha\alpha$ -Tm regulated filaments and yet there was never a significant effect from phosphate on acetyl serine 1 of TnT (or fragment T1), which suggests that higher levels of phosphorylated Tm may actually be able to produce more rapid contractions [Heeley, 1994].

Quantifying Post-Translational Modifications with Mass Spectrometry

High pressure liquid chromatography coupled with electrospray ionization tandem mass spectrometry can determine the levels of post-translational modifications in protein samples such as native thin filaments (NTF) [Previs et al., 2008]. Liquid chromatography involves the separation of molecules via passing a sample in solution through a column that has different affinities for certain functional groups or other characteristics of the molecules. For our study we used hydrophobic interaction chromatography (HIC) and

thus we used a column (C18) designed to interact with the hydrophobic residues in our sample peptides [Weith et al., 2012]. During chromatography, a column is loaded with the sample and then the solution is slowly changed over a gradient to allow proteins to be separated slowly over time, which allows an undesired "void" volume to be washed out of the column before the sample peptide.

During electrospray ionization, a high voltage current is applied to samples to vaporize them into ions, which can then be separated by their mass-to-charge ratio via the electromagnetic fields within the mass spectrometer. These ions can be quantified and the ion profiles compared to a library of known protein products. The amount of phosphorylation can be determined by comparing the ion currents from each desired peptide sequence in a phosphorylated set to its dephosphorylated reference peptide using a mass balance approach [Previs et al., 2008 , Weith et al., 2012]. Two sets, one with and one without dephosphorylation by alkaline phosphatase, are required to quantify a change to the peptides phosphorylation status. This is due to phosphorylation causing mis-cleavage when near tryptic cleavage sites [Steen et al., 2005], which otherwise would alter the results from the mass spectrometry.

Summary

Ischemia has been proven to induce a clear decrease in tension related to decreased Ca^{2+} sensitivity, which implicates alterations to the cardiac regulatory proteins as well as the myosin in terms of their interactions with actin. There are several damaging compounds which can accumulate during periods of restricted blood flow and might cause deleterious modifications to the regulatory proteins and possibly myosin as well. For example myosin was found to have some reactive cysteines oxidized due to ischemia

and any alterations to these areas could lead to a loss of function, but there are conflicting data as to whether myosin is a primary cause of decreased function. Myosin binding protein -C was also implicated, because it had an increase in carbonylation due to oxidative stress in the tissues remote to localized heart tissue infarct correlated with decreased function. Changes in phosphorylation status of TnI could be a key factor in Ca^{2+} controlled actomyosin interaction, because of its interaction with TnC (the subunit that binds Ca^{2+}). Also Tm, from skeletal muscle, was found to have an increase in phosphorylation, but dissimilar to Tn phosphorylation it produced more rapid contractions.

Although there has been extensive research attempting to elucidate the factors that cause changes in tension and contractile properties of post-ischemic cardiac tissue, there is still no specific understanding of the direct mechanism of this change. There are many leading theories involving oxidative damage or metabolite build up that could lead to covalent modifications to regulatory proteins or to myosin itself and thus reduce force production. The specific mechanism causing reduced Ca^{2+} sensitivity remains unknown, but it may be that the Ca^{2+} binding subunit TnC loses affinity for Ca^{2+} upon direct phosphorylation or phosphorylation of another subunit. Differences in animal models can be especially important and thus porcine post-ischemic and reperfusion models should be studied to understand tissues very similar to humans. One of the most important tools to employ in these studies is the use of *in vitro* motility assays specifically designed to examine the differences in post-ischemic and reperfused myosin and NTF (actin, Tn, and Tm). The actual change to the actomyosin crossbridge cycle is also unknown and without knowledge of the affected proteins it is difficult to pinpoint the specific alterations in the

cycle. Mass spectrometry can accurately detect the post translational modifications to the proteins and thus can suggest underlying mechanisms for observed functional changes. It remains unanswered if there is permanent ischemia and reperfusion damage or if there are therapies that can reverse the changes which occurred to the protein structures. Research needs to be conducted to discover the protein alterations and thus the mechanisms by which reduced left ventricle function occurs before more effective treatments can be discovered.

CHAPTER III

METHODS

Ischemia/reperfusion protocol

All surgical procedures involving animals were in strict compliance with the NIH Guide for the Care and Use of Animals. The parent study from which the tissue samples were obtained was IACUC-approved and conducted in a USDA certified laboratory. Female domestic Yorkshire swine aged 3–4 months and weighing approximately 30–35 kg prepared as previously described [Mader et al., 2010], were used for the cardiac arrest-resuscitation experiments. The three animals included in this substudy were part of a larger study involving 80 animals, 45% of which attained ROSC. During the protocol the animals were sedated with intramuscular telazol (5 mg/kg), ketamine (2.5 mg/kg), and xylazine (2.5 mg/kg). Isoflurane was provided to facilitate endotracheal intubation and intravenous (IV) access. The inhalation anesthetic was then discontinued and a surgical plane of anesthesia was achieved using an IV propofol bolus (2 mg/kg) followed by a continuous infusion (80 mcg/kg/min) titrated to effect. The animals were ventilated with room air, using a volume-cycled ventilator adjusted the tidal volume and ventilatory rate to maintain eucapnea. A nasopharyngeal probe was placed through the oral cavity into the animal's esophagus to measure core body temperature. Three surface electrodes configured to correspond to a standard lead II electrocardiogram (ECG) surface electrodes were secured to the proximal forelimbs and thorax.

Neuromuscular paralysis was induced with pancuronium (4 mg initial bolus IV) and an arterial introducer (8.5 Fr) was placed into the right femoral artery and a venous introducer (8.5 Fr) into the right femoral vein under direct visualization. Micro–

manometer tipped pressure catheters (Mikro-Tip, Millar Instruments, Houston, TX) were placed into the ascending aorta and right atrium. Arterial blood gas was obtained as soon as access was established and just prior to ventricular fibrillation induction. All central vascular access ports were connected to a pressurized liter bag of normal saline containing heparin. The ECG tracing, as well as the arterial and venous pressures were monitored and recorded continuously throughout the experiment (PowerLab M8/30, AD Instruments, Colorado Springs, CO). Immediately before induction of ventricular fibrillation, a 2-mg bolus of pancuronium was given, the propofol infusion was discontinued, and the ventilator was disconnected.

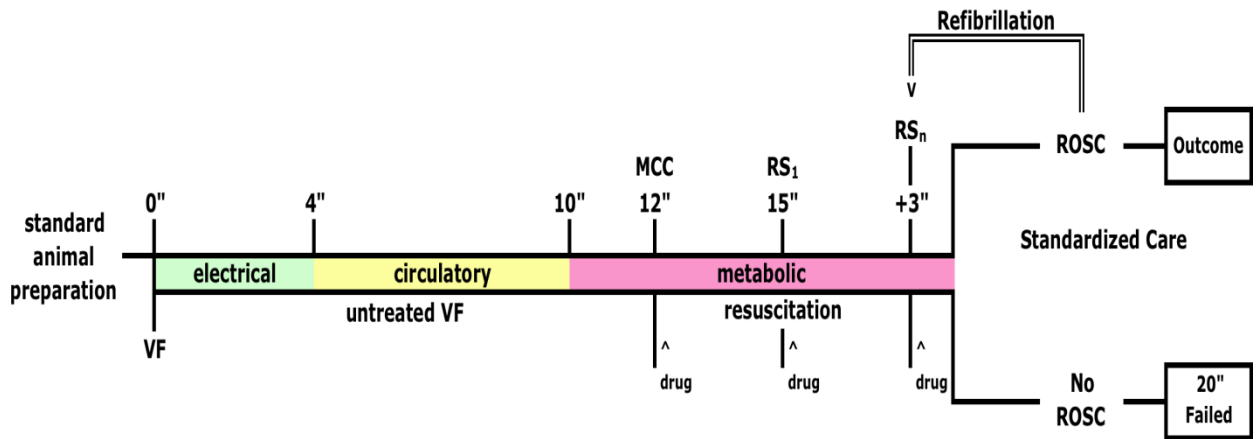


Figure 5. Global ischemia protocol. Ventricular fibrillation was induced as previously described [Mader et al., 2010] with a transthoracic current (100mA at 60Hz) and was untreated for 12min. ventricular fibrillation lasted for 4min before complete electrical failure was reached (green bar) at which point circulation stopped completely (yellow bar) and finally metabolic failure was reached at ~10min. (pink bar). Resuscitation efforts began 12min after induction of ventricular fibrillation, initially with manual chest

compressions (MCC) and infusion of epinephrine (drug, 0.1mg/kg). At minute 15 we attempted to restart circulation electrically (RS1) and at 18min. If ROSC it was maintained for 2hrs and then the animals were sacrificed and cardiac muscle samples were taken from the left ventricle (IR2). If ROSC was not restored then after 20min cardiac muscle samples were taken from the LV (IDNR). Control animals were prepared identically but did not receive the ischemia protocol (Control). “~” Indicates when a drug was administered.

Global ischemia and the resuscitation protocol were performed as previously described [Mader et al., 2010]. Briefly, ventricular fibrillation was induced by a 3-s, 60-Hz, 100-mA transthoracic alternating current. At minute 12 of untreated ventricular fibrillation, dynamic baseline characteristics were again recorded and resuscitation was attempted beginning with mechanical chest compressions using an oxygen-powered mechanical resuscitation device (Life-Stat Mechanical CPR System, Michigan Instruments, Grand Rapids, MI) that provides standardized closed chest compressions in the anterior-posterior direction at a rate of 100/min. The device was programmed to deliver chest compressions and ventilation ($V_t = 500$ cc, $FiO_2 = 100\%$) in a ratio of 30:2.

The animals were resuscitated using a standard combination of resuscitation drugs [Fig 5]. Attempted resuscitation was terminated after the ROSC or 20 min of failure. Animals attaining ROSC were immediately placed back on the ventilator, a low dose propofol infusion was restarted and titrated to optimize the effect, and norepinephrine was given intravenously to maintain a systolic blood pressure above 80 mm Hg for 2 h.

At the conclusion of each experiment, the hearts were immediately excised, diced, and put into liquid nitrogen. The samples were kept on dry ice during transport and stored in a -80°C freezer.

Cardiac tissue samples were obtained from porcine hearts that underwent one of three conditions: (1) samples from a control animal that was subjected to the surgery but that did not experience cardiac arrest or subsequent resuscitation and reperfusion (control); (2) from an animal subjected to 12 min of global ischemia and attempted resuscitation for 20 min that included epinephrine (0.01 mg/kg), vasopressin (0.5 U/kg), amiodarone (4 mg/kg), sodium bicarbonate (1.0 mEq/kg), and metoprolol (0.2 mg/kg) but spontaneous circulation failed to return (IDNR); and (3) from an animal that underwent 12 min of global ischemia and successful resuscitation after epinephrine (0.01 mg/kg), vasopressin (0.5 U/kg), amiodarone (4 mg/kg) on the third defibrillation attempt, followed by 2 h of sustained reperfusion (IR2).

Proteins

Cardiac myosin was isolated from porcine ventricular tissue based on a method previously established for mouse cardiac tissue [Tyska et al., 2000]. The purification of the myosin molecules involves the manipulation of salt concentration of the extraction solution, which at low salt the myosin monomers form filaments that can allow them to be isolated from the other tissue proteins by ultracentrifugation. Briefly a 2-mL aliquot of extraction buffer (300 mM KCl, 150 mM PO_4 , 20 mM EDTA, 5 mM MgCl_2 , pH 6.7, 3.3 mM ATP, and 5 mM DTT) was added to ~400 mg of porcine cardiac tissue and continuously homogenized for 12 minutes in a 2mL dounce homogenizer. The dounce was chilled prior to introduction of the tissue as well as kept submerged in ice for the

majority of the 12 minutes of homogenizing. The solution was then centrifuged (5 minutes; 10,000 g; 4 °C) to pellet insoluble tissue and the supernatant was centrifuged once more for 20 minutes at 400,000 g to remove any filamentous proteins. The myosin containing supernatant was then precipitated by 10-fold dilution with ddH₂O for 60 minutes and then centrifuged for 10 minutes at 10,000 g, and 4 °C. The supernatant was discarded and the myosin pellet was gently rinsed with cold ddH₂O. The pellet was homogenized in a minimum volume of a high salt buffer (25 mM Imidazole, 600 mM KCl, 1 mM EGTA, 4 mM MgCl₂, pH 7.4) and the concentration determined with a spectrophotometer (extinction coefficient of 0.55, [Margossian and Lowey, 1982]).

An additional purification was performed on a subset of the cardiac myosin using HIC as previously described [Malmqvist et al., 2004] with minor modifications. Briefly, the isolated myosin was first dialyzed overnight at 4 °C against 1.45 M ammonium sulfate (AmSO₄) and then centrifuged (15 minutes; 10,000 g) to remove any aggregating proteins. The supernatant was loaded on to the HIC column (5 mL Toyo pearl ether-650M, Tosoh Biosciences Inc., Grove City, OH) at 1.45 M AmSO₄. The AmSO₄ was reduced to 1.2 M causing the elution of the purified myosin, following a collection of proteins that weakly interacted with the column (Figure 7 shows chromatograph and gel).

NTF were isolated using an established protocol [Lehman et al., 1995], in which ~200 mg of tissue was exposed to thin filament extraction buffer (25 mM imidazole, 1 mM EGTA, 100 mM KCl, 4 mM MgCl₂, 5 mM ATP, 10 mM DTT, pH 7.0) and homogenized for 12 min on ice (0°C). The homogenate was initially centrifuged (10,000 g, 4°C) for 5 min followed by a second centrifugation (40,000 g, 4°C) of the supernatant for 20 min to remove myosin and any bulk tissue. Subsequently, the supernatant was kept

and centrifuged (200,000 g, 4°C) again for 45 min to pellet the thin filaments which were re-suspended in 300 µL of thin filament extraction buffer before a final clarification spin (40,000 g, 4°C) for 5 min to remove any remaining myosin. The supernatant was kept and then a final centrifugation was performed for 45 min at 200,000 g to collect the thin filaments. The resultant pellets were brought up in an extraction buffer (7.0 pH, 25 mM imidazole, 1 mM EGTA, 100 mM KCl, 4 mM MgCl₂, 10 mM DTT) labeled tetramethylrhodamine isothiocyanate-phalloidin (TRITC-phalloidin) (Sigma-Aldrich Inc., St. Louis, MI) at a 1 µM. Chicken skeletal actin was also used to assess the cardiac myosin function and it was isolated from pectoralis muscle and fluorescently labeled with TRITC-phalloidin.

Solutions

The pH of each buffer was determined by a Fisher Scientific pH-meter, which was calibrated prior to determining the pH of each solution. Sodium hydroxide (NaOH) was used to increase the pH of each motility solution and hydrochloric acid (HCl) was used to decrease it.

Immediately prior to beginning a motility experiment, the myosin was further purified by removing myosin heads that were trapped in a rigor state and thus inactive. This involved the addition of filamentous chicken skeletal actin and 2mM ATP and centrifugation at 400,000g for 20 minutes at 4°C. After which the supernatant was introduced into the flow cells in the motility assay. The rigor-like myosin heads would remain bound to the filamentous actin in solution, but the functional myosin heads would hydrolyze the ATP, separate from the filamentous actin, and would remain in the supernatant solution.

In vitro motility

Flow cells were used to inject different elements that were needed to simulate physiological environments and to study the motility of molecular motor proteins. This involves nitrocellulose coated coverslips glued onto a glass slide with enough space for the injection of solutions. The technique was created to view moving fluorescent actin filaments in the presence of ATP on a bed of myosin, which is affixed to glass [Kron and Spudich, 1986]. The flow cell temperature was maintained at 30°C with an objective temperature controller collar.

The impact of the ischemia/reperfusion of contractile protein function was assessed using the *in vitro* motility assay using previously established methods [Debold et al., 2011, Debold et al., 2012]. Briefly, 60uL of isolated myosin (in a high salt buffer: 300 mM KCl, 25 mM Imidazole, 1 mM EGTA, 4 mM MgCl₂, pH 7.4, 10 mM DTT) was adhered to a nitrocellulose coated microscope coverslip as part of a flow cell at 200ug/ml and incubated for 30 seconds. 60uL of bovine serum albumin (BSA) (0.5mg/mL) was then added to cover any areas of the surface not coated by myosin and allowed to incubate for 60 seconds. Then 60uL of actin coat (actin that had been put onto a vortex mixer to ensure only small actin filaments would be present for the myosin heads to bind to) in the absence of ATP, was injected into the flow cell and incubated for 30 seconds. 40uL of ATP wash was injected into the flow cell and then incubated for 30 seconds, which was repeated immediately after the initial incubation for a total of 80uL. This allowed all functional myosin heads to release the small actin coat pieces. 40uL of TRITC-labeled NTF (5nM in actin buffer) was then injected and incubated for 60 seconds, which was repeated immediately after the initial incubation for a total of 80uL.

Then 60uL of actin buffer was flowed through the cells, which maintained a low salt environment for the actin. 60uL of the final motility buffer was added to the chamber (25 mM KCl, 25 mM Imidazole, 1 mM EGTA, 4 mM MgCl₂, pH 7.4, 2 mM ATP, 10 mM DTT, with an amount of CaCl₂ required to achieve the appropriate free [Ca²⁺]), which also contained an oxygen scavenging system (glucose, glucose oxidase, and catalase) to slow photo-bleaching of the fluorescently labeled actin filaments.

The flow cells were then loaded onto a Nikon Eclipse Ti inverted microscope with a 100X, 1.4NA CFI Plan Apo objective and the fluorescent actin filaments were visualized by an ICCD camera (Stanford Photonics, Inc., Palo Alto, CA). The video was captured by an Epix-LVDS frame grabber (Epix, Inc., Buffalo Grove, IL) coupled to the ICCD camera. Piper Control™ 2.5 software (Stanford Photonics, Inc. Palo Alto, CA) was used to capture three to four video fields for each flow cell at 10 frames s⁻¹ for 30 seconds. The filament motions were manually tracked using the MtrackJ [Meijering et al., 2012] plugin for ImageJ [Abramoff et al., 2004]. The percentages of filaments moving were calculated via an ImageJ plug-in called wrMTrck [Nussbaum-Krammer et al., 2015]. 4-5 motility experiments were conducted each using fresh isolations for both myosin and thin filaments for each condition.

Differences in naked skeletal actin and NTF velocities among each condition were determined using a One-Way ANOVA followed by a Tukey's HSD *post hoc*. To determine the Ca²⁺ sensitivity of the NTF, their velocities as a function of the -log of the Ca²⁺ concentration (pCa) were fitted to the Hill equation:

$$V = V_{\max}/(1 + 10^{n(pCa_{50} - pCa)})$$

using SigmaPlot R 11.2 (Systat Software, San Jose, CA). The hill equation derived the Ca^{2+} concentration required to elicit half maximal filament velocity (pCa50) and the Hill coefficient (n) to gain insight into the cooperative behavior of activation.

Mass Spectrometry

In preparation for mass spectrometry denaturing SDS-PAGE was conducted with precast 12% bis-Tris polyacrylimide gels (BioRad, Life Science Research, Hercules, CA). The identified bands were excised from the gel, cut into small cubes, and placed into Eppendorf tubes. Residual stain and excess water was removed with a 50% acetonitrile solution and the gel slices were dried in a speed vacuum device as previously described [Previs et al., 2012]. Next, one set of samples was rehydrated with 8 μL of alkaline phosphatase (Sigma–Aldrich Inc., St. Louis, MI) in 92 μL of ammonium bicarbonate and incubated for 18 h at 30°C, allowing the phosphatase to thoroughly impregnate the gel and dephosphorylate the protein samples. The samples were dried via a speed vacuum device and subsequently rehydrated with 2 μg of trypsin (Promega, Madison, WI) in 100 μL of ammonium bicarbonate and incubated for 18 h at 37°C. After 18 h of incubation 7 μL of 90% formic acid was added to deactivate the trypsin. The resultant peptides were extracted with 25 mM ammonium bicarbonate/50% acetonitrile solution, dried in a speed vacuum device, and reconstituted in 0.05% heptafluorobutyric acid.

Electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS) was carried out in data dependent mass spectrometry mode using an LTQ ion trap mass spectrometer (Thermo Electron Corporation) coupled to a 1 mm C18 column as previously described [Weith et al., 2012]. Initial SEQUEST searches were performed to

identify peptides using the IPI human protein sequence database (v3.75) downloaded from the EMBL- EBI website. Subsequent searches only contained the pig cardiac TnI (A5X497) and pig α -Tm (P42639) sequences downloaded from UniProtKB. The degrees of site-specific TnI and α -Tm phosphorylation and truncation of the C-terminus of TnI were determined from the extracted ion currents for specific peptides of interest in the LC chromatograms using label free-proteomic strategies as described in the Results Section. Phosphorylation levels were determined using each of the five reference peptides listed for TnI and α -Tm in the Results Section in three samples from each group, before and after treatment with alkaline phosphatase. Statistical significance was determined from the individual measurements from each reference peptide using a Student's t-test.

CHAPTER IV

RESULTS

Changes to Contractile Protein Function

Cardiac myosin's ability to translocate actin filaments *in vitro* was not significantly different in samples that underwent either ischemia with (IR2) or without reperfusion due to failed resuscitation (IDNR) [Fig 6]. This indicated that the ischemia/reperfusion protocol may not have had an effect on the myosin's function.

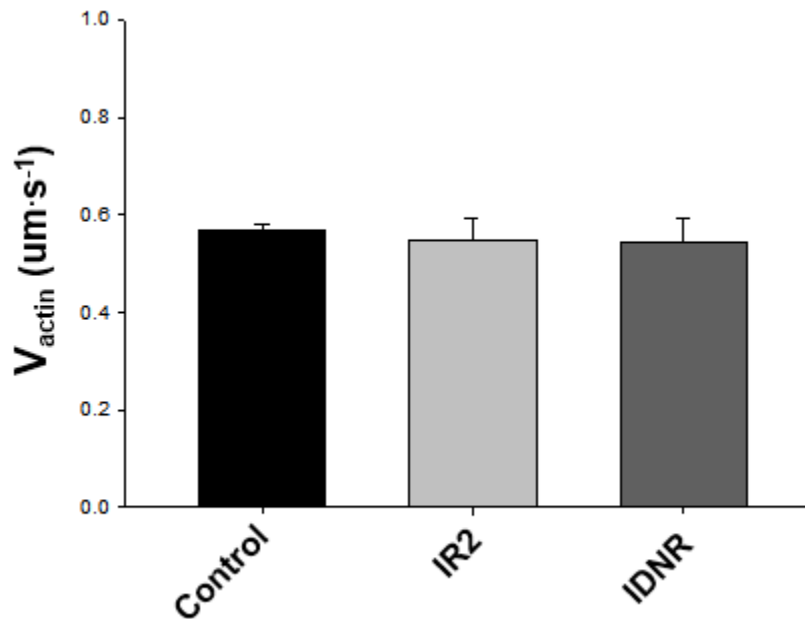


Figure 6. Effect of ischemia/reperfusion on V_{actin} . Means \pm SEM for unregulated (no Tn/Tm) actin filament velocities in the *in vitro* motility assay using myosin isolated from the corresponding condition. The myosin used was isolated from tissue from control, ischemia with 2 hours of reperfusion (IR2), and ischemia without resuscitation (IDNR). Data were analyzed using a Kruskal-Wallis ANOVA, but all comparisons were non-significant ($p > 0.05$).

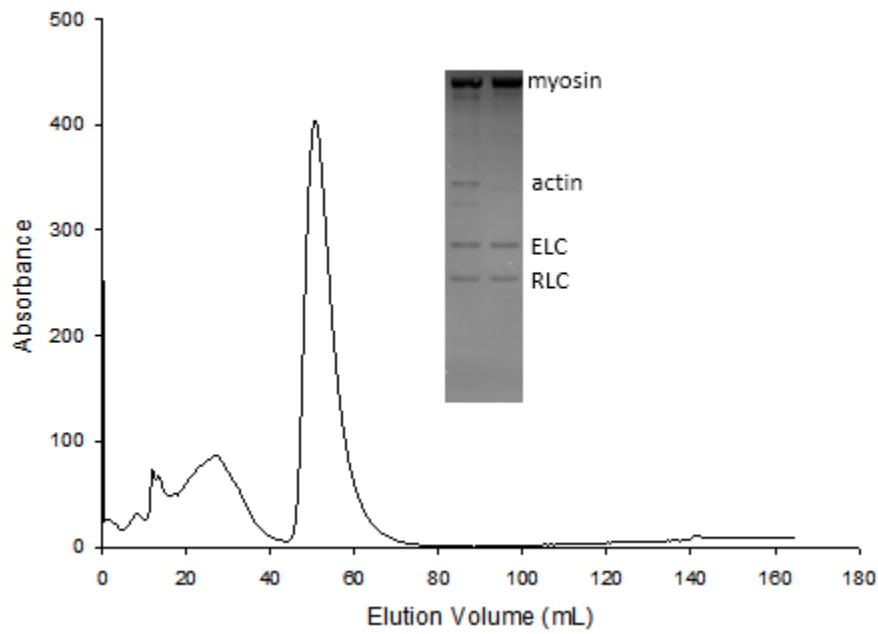


Figure 7. Representative chromatograph from myosin purification over an HIC column. The isolated myosin from porcine myocardium was additionally purified using HIC based on a previously described methodology [Malmqvist et al., 2004] with minor modifications (see Methods). Chromatograph displays absorbance at 280nm vs. elution volume. Proteins with minimal affinity for the column elute at high (1.45M) AmSO₄ (first broad peak). After the AmSO₄ is reduced to 1.2M myosin is released (large narrow peak). This process removed impurities including actin and likely Tm, selecting for myosin and its light chains (Essential Light Chain, Regulatory Light Chain) as shown by SDS-PAGE gel (inset).

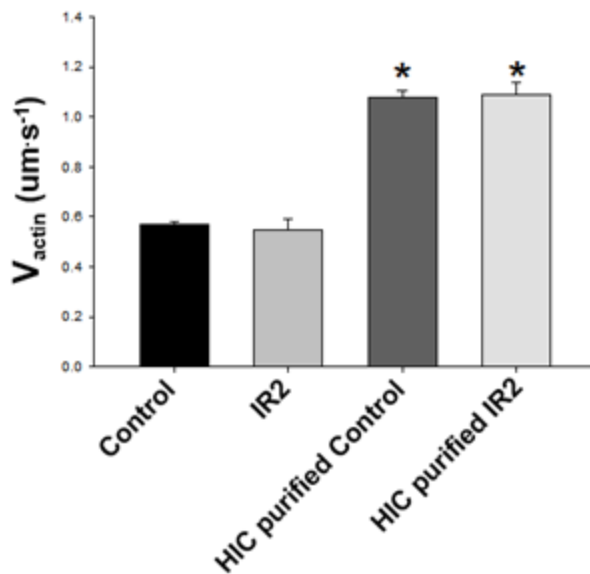


Figure 8. Purification with HIC column enhances V_{actin} . Further purification of the myosin using an HIC column nearly doubled V_{actin} from both Control tissue and tissue exposed to ischemia with 2 hours of reperfusion (IR2) ($P<0.001$). However, there was no difference between conditions with either the isolated myosin or the HIC purified myosin.

Cardiac myosin was purified via an HIC column as it may be less stable than skeletal myosin and possibly the ischemia increased this instability. Control and IR2 myosin samples were run over the column [Fig 7] and the resultant data displayed that the purification enhanced velocity to nearly double the velocity of both samples [Fig 8]. However, both samples were improved and were not significantly different from each other, which suggests that the column likely removed impurities and myosin that poorly hydrolyzes ATP [Malmqvist et al., 2004] in earlier peaks than the eluted myosin sample.

The proteins eluted before the myosin sample were present in all samples, but the quantity was not affected by the ischemia/reperfusion protocol. It appeared that myosin's function in the *in vitro* motility assay was unaffected by our model of ischemia/reperfusion.

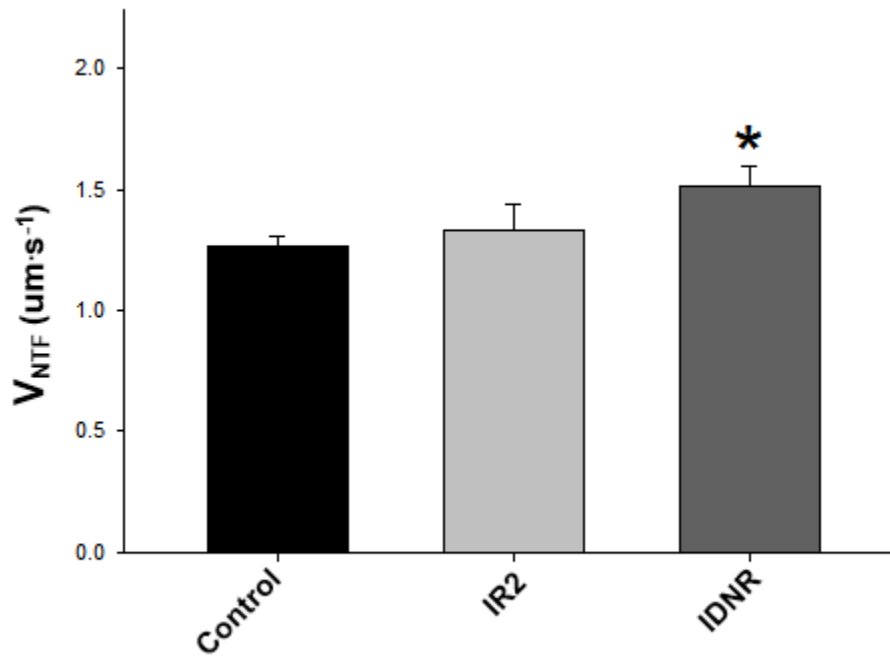


Figure 9. Effect of ischemia/reperfusion on NTF velocities (V_{NTF}). Means \pm SEM for native thin filament velocities in the *in vitro* motility assay using myosin isolated from the control tissue sample. The NTF used was isolated from control, ischemia with 2 h of reperfusion (IR2), and ischemia without resuscitation (IDNR). Data analyzed using a non-parametric Kruskal–Wallis ANOVA and indicated that IDNR was significantly greater than control velocity *, ($p < 0.05$). The data represent the average actin filament velocities from 20 to 30 s videos from Control, 19 from IR2 and 16 from IDNR.

Regulatory proteins may be vulnerable during ischemia [Gao et al., 1997 , McDonough et al., 1999] and so we determined the velocity of these NTF isolated from each condition. At pCa 4 control and IR2 NTF samples had identical velocities [Fig 9 , Fig 10], however IDNR samples moved significantly faster at 25% ($p < 0.05$) greater than the control value [Fig 9]. The increased average velocity cannot be due to a decrease in moving filaments, because the percentage of moving filaments was similar for each condition. This suggests that the IDNR condition had some modification to the regulatory proteins Tn/Tm that regulate myosin's interaction with actin

This regulation is Ca^{2+} dependent, so one can measure the velocity as a function of Ca^{2+} and so we determined the Ca^{2+} sensitivity [Fig 10], which was plotted as the concentration of free Ca^{2+} in solution expressed as pCa. The IDNR NTF sample had a significantly increased Ca^{2+} sensitivity ($\text{pCa}_{50} 6.57 \pm 0.04$) compared to the control (6.34 ± 0.07) and IR2 (6.47 ± 0.13) [Fig 10A , Table 1]. pCa_{50} is a measure of the Ca^{2+} concentration required to achieve half maximal activation of the filament and the IDNR sample had an increased pCa_{50} , because it activated at a lower Ca^{2+} concentration than the other 2 samples and thus is more “sensitive” to $[\text{Ca}^{2+}]$. Although not statistically significant, the Hill coefficient was increased in IDNR samples, which suggested the myosin and IDNR NTF sample seemed to have more cooperative binding compared to control filaments. A higher Hill coefficient suggested that there is a cooperative relationship between the binding of the ligand (exposed myosin binding site on actin) and its enzyme (myosin) and a subsequent increase in the affinity of the enzyme for the ligand (increased likelihood of a separate myosin molecule to interact with actin). We then used quantitative mass spectrometry to determine what structural modifications may

have occurred to Tn or Tm to induce this significant functional change only in the IDNR sample.

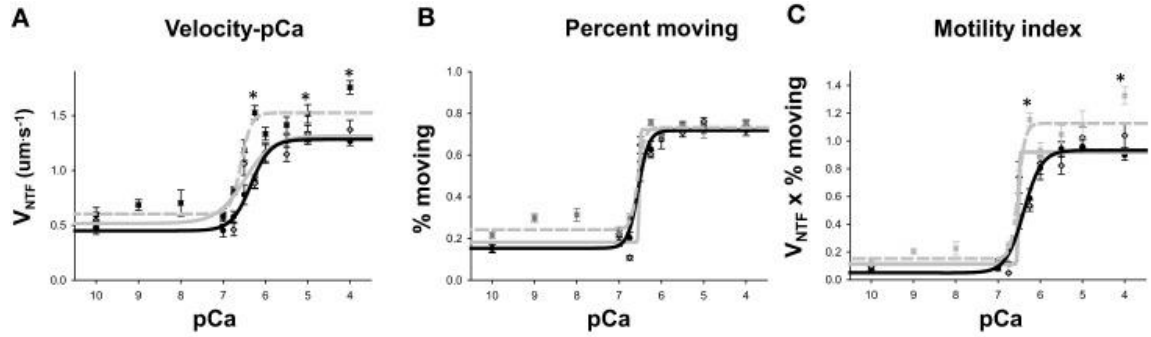


Figure 10. Velocity-pCa data. A, NTF velocity (V_{NTF}) plotted as a function of free $[\text{Ca}^{2+}]$ in $-\log$ units (pCa). Thin filaments from Control tissue are plotted with filled black dots and solid line, IR2 thin filaments with gray diamonds and solid gray line and the filaments from IDNR are plotted with dark gray boxes and a dashed gray line. Points represent mean \pm SEM and the data were fit with the Hill equation (see Methods). * indicates significantly ($p < 0.05$) different from WT V_{NTF} . B, percentage of NTF moving as a function of free $[\text{Ca}^{2+}]$ symbols and lines same as in A. C, Motility index, defined as the product of V_{NTF} and percent moving plotted as a function of free $[\text{Ca}^{2+}]$.

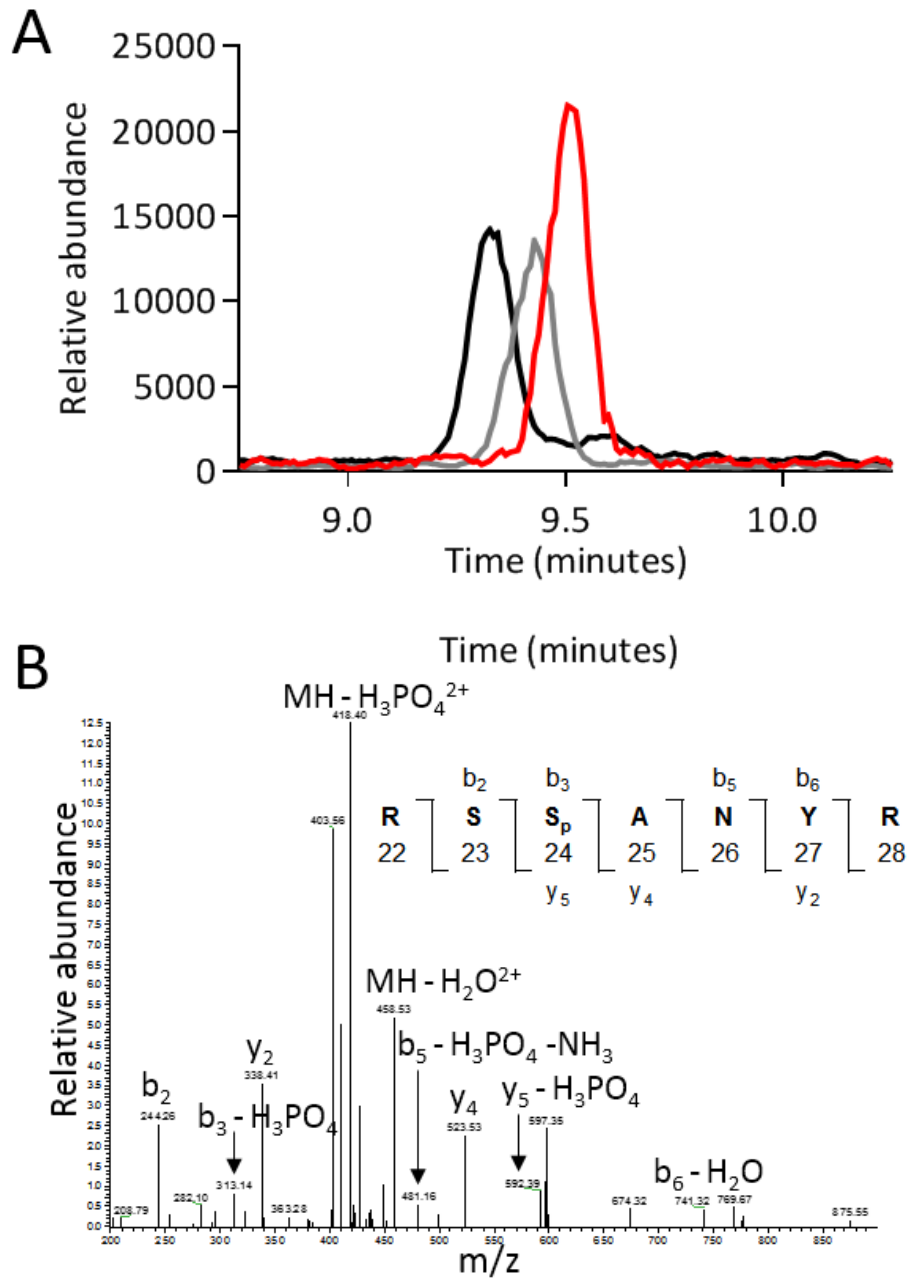
V_{NTF}	pCa_{50}	n
Control	6.34 ± 0.07	1.89 ± 0.48
IR2	6.47 ± 0.13	1.27 ± 0.42
IDNR	$6.57 \pm 0.04^*$	3.46 ± 1.00

Table 1. Parameters for velocity-pCa fits to the Hill eq. V_{NTF} indicates NTF velocities for filaments isolated from non-ischemic control, ischemia followed by 2 hrs of reperfusion (IR2) and ischemia followed by failed resuscitation (IDNR). n represents the Hill coefficient. Values represent mean \pm SEM. * indicates significantly ($p < 0.05$) different from Control tissue.

No Truncation of Troponin I

One of the most frequently observed structural changes associated with ischemia is the proteolytic cleavage of the C-terminal end of TnI [Westfall and Solaro, 1992 , Gao et al., 1997 , van Eyk et al., 1998 , McDonough et al., 1999 , van Eyk and Murphy, 2001]. We therefore used a mass spectrometry based, mass-balance approach to determine if the C-terminus of TnI was truncated during ischemia. We measured the relative abundance of the sum of the C-terminal 195NIDALSGMEGR205 ($m/z = 581.78$) and 195NIDALSGm*EGR205 ($m/z = 589.77$) peptides in the control and experimental samples that would be removed by ischemia induced proteolytic cleavage. The area under the LC peak for the C-terminal peptide was normalized using the TnI reference peptides used for the quantification of phosphorylation. The abundance of the C-terminal peptide

did not differ for either group [1.09 ± 0.19 (IR2), 1.06 ± 0.26 (IDNR)] when compared to controls. This lack of difference suggests that the C-terminus of TnI was not truncated as a result of the ischemic treatments employed in the present study.



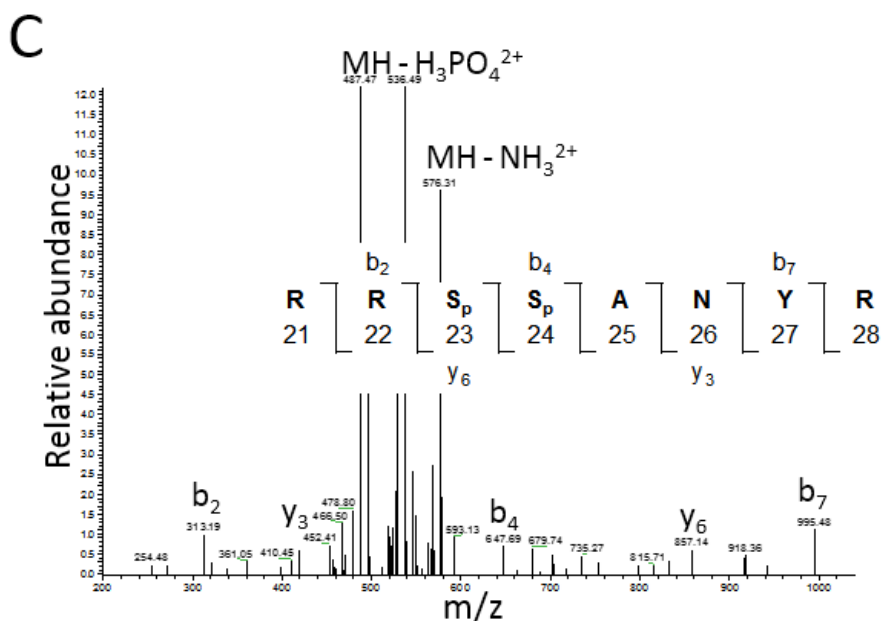


Figure 11. LC elution profiles and data dependent MS₂ spectra for TnI peptides. A, Representative LC elution profiles for the non-phosphorylated 23SSANYR28 (red), mono-phosphorylated 22RSSpANYR28 (grey) and bis-phosphorylated 21RRSpSpANYR28 (black) peptides. Data dependent MS₂ spectra for the B, mono-phosphorylated 22RSSpANYR28 and C, bis-phosphorylated 21RRSpSpANYR28 peptides showing fragment ions used for peptide identification and localization of phosphate.

Quantification of Phosphorylation

Peptides containing phosphate including: the mono-phosphorylated 22RSSpANYR28 ($m/z = 467.20R$) and bis-phosphorylated 21RRSpSpANYR28 ($m/z = 585.23$) peptides coming from TnI [Fig 11]; and 269AISEELDHALNDMTSpI284 ($m/z = 919.89$), 269AISEELDHALNDm*TSpI284 ($m/z = 927.89$), and

269AISEELDHALNDm⁺TSPI284 ($m/z = 935.89$) peptides coming from α -Tm were identified in the data dependent MS2 spectra and manually confirmed [Previs et al., 2008 , Weith et al., 2012]. (It should be noted that Sp represents phosphoserine and m* and m⁺ denote methionine sulfoxide and sulphone). The degree of phosphorylation at these sites was determined via a mass-balance approach [Previs et al., 2008 , Weith et al., 2012] from the abundance of the non-phosphorylated analogues of these phosphopeptides in each sample prior to and after the removal of phosphate with alkaline phosphatase. The non-phosphorylated analogues were the properly cleaved 23SSANYR28 peptide and the sum of the ion currents for the 269AISEELDHALNDMTSI284, 269AISEELDHALNDm*TSI284 and 269AISEELDHALNDm⁺TSI284 peptides. Whereas this mass-balance approach is an indirect way to measure phosphorylation levels, it provides accurate quantification of the fraction of the protein molecules in a sample which are phosphorylated and obviates problems with quantification arising from mis-cleavage when the phosphate is proximal to the tryptic cleavage site [Previs et al., 2008]. For quantification, measured ion currents corresponding to peptides of interest were extracted from the LC chromatogram and the area under each LC peak was normalized using reference peptides within each sample to account for difference in the total amount of protein loaded onto the gel [Previs et al., 2008 , Weith et al., 2012]. The area under each LC peak for the TnI peptides of interest were normalized using the YDVEAK($m/z = 362.67$), KLQLK ($m/z = 629.43$), ETLDLR ($m/z = 746.40$), NITEIADLNQK($m/z = 629.83$), and IFDLR ($m/z = 332.19$) peptides; and the area under each LC for α -Tm peptides of interest were normalized using the HIAEDADR($m/z =$

463.72), SLEAQAEK ($m/z = 438.22$), IQLVEEELDR ($m/z = 622.33$), LVIIESDLER ($m/z = 593.83$), and SIDDLEDELYAQK ($m/z = 769.86$) peptides as references.

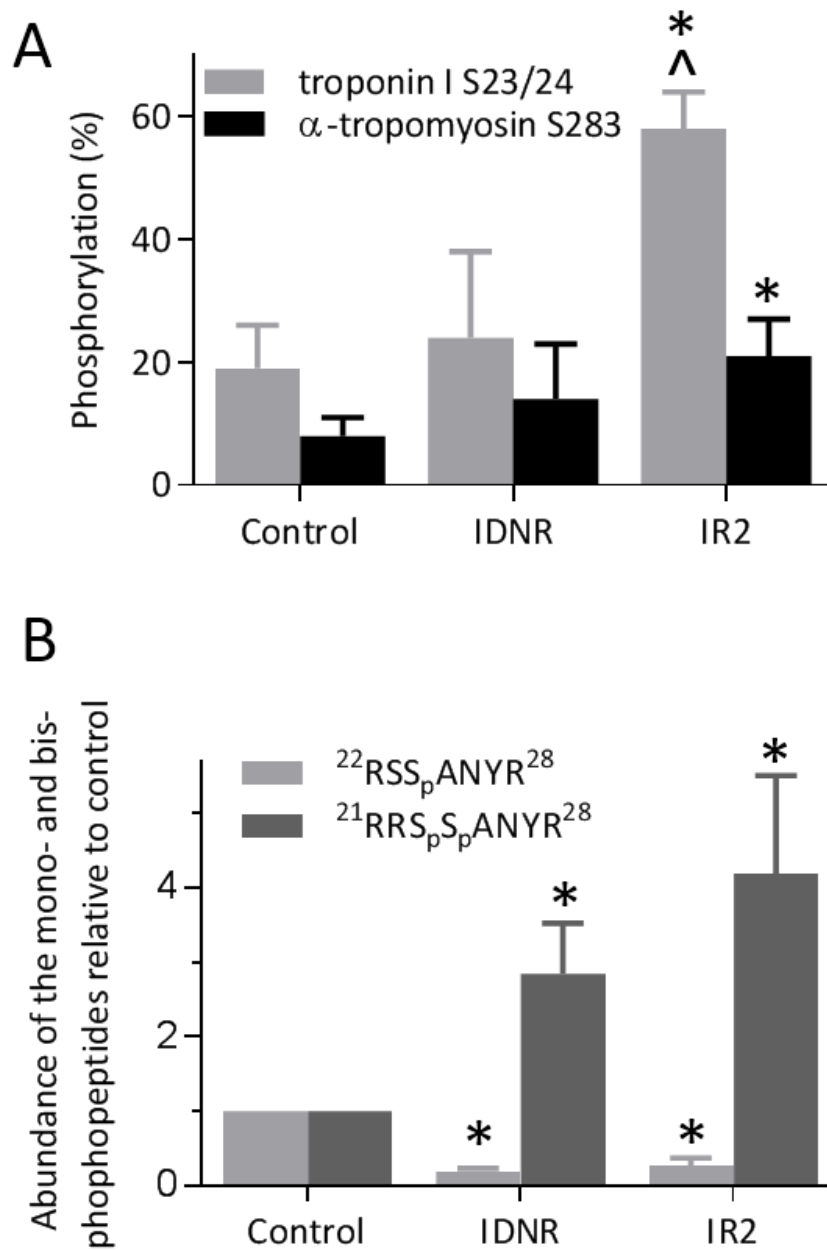


Figure 12. Quantification of TnI and α-Tm phosphorylation. A, Percent phosphorylation of TnI serines 23 and/or 24 and Tm serine 283 in NTF isolated from

control hearts, and hearts following global ischemia that did not survive attempted resuscitation (IDNR) or survived and were then subject to 2 hours of reperfusion (IR2).

B, Levels of mono- (serine 24) and bis-phosphorylated (serines 23 and 24) TnI relative to the control. * $p < 0.01$ relative to control and ^ $p < 0.01$ relative to IDNR.

The overall percent phosphorylation of both TnI at serines 23/24 and α Tm at serine 283 was significantly enhanced with respect to the control [Fig 12A] in samples from successful resuscitation followed by 2 hours of reperfusion (IR2). In contrast, the overall levels of TnI and Tm phosphorylation of samples from the failed resuscitation (IDNR) did not significantly differ from the control samples [Fig 12A]. However, this method of quantification is not sensitive to changes in site-specific phosphorylation when multiple phosphorylation sites are located within a single peptide, as was the case for TnI serine 23/24. Therefore, we determined if there was a shift in the phosphorylation profile between the mono- and bis-phosphorylated states by calculating the relative abundance of the mono-phosphorylated 22RSSpANYR28 and bis-phosphorylated 21RRSpSpANYR28 peptides in each sample [Fig 12B]. We observed significant reductions in the abundance of the mono-phosphorylated peptides (0.27 ± 0.10 (IR2) and 0.19 ± 0.04 (IDNR)) and corresponding increases in the abundance of the bis-phosphorylated peptides (4.2 ± 1.3 (IR2) and 2.8 ± 0.7 (IDNR)) with respect to the controls following the bout of ischemia regardless of survival [Fig 12B]. Therefore, under both ischemic conditions we observed an increase in the relative abundance of the bis-phosphorylated TnI 21RRSpSpANYR28 peptide with respect to the control.

CHAPTER V

DISCUSSION

***In vitro* Function of the Contractile Proteins**

This study attempted to discover what structural and functional effects occurred to the major contractile proteins following global myocardial ischemia and reperfusion. The *in vitro* motility assay revealed no difference in labeled actin filament velocity for cardiac myosin samples in either IR2 or IDNR conditions compared to the control [Fig 6]. As there was no difference in myosin's ability to translocate actin, any modifications present were unlikely to have caused the depressed function others have seen in different studies. Thus no further experiments were performed to determine if there were any structural modifications to myosin. Other studies have used different ischemia paradigms, such as ligation of blood vessels inducing local ischemia, which has been shown to cause modifications by the production of ROS during ischemia [Zweier et al., 1989]. The ROS can cause modifications to contractile proteins [Bolli and Marban, 1999] and affect myosin's ATPase [Tiago et al., 2006] and the ability to produce force *in vitro* and translocate actin [Rao et al., 2007]. Other studies also employed longer periods of ischemia (up to 60 minutes) and subsequent reperfusion of the heart for 24-72 hours. The different protocols for inducing ischemia, as well as the durations of induced ischemia and reperfusion could be the reason for the lack of observed changes to myosin. This study had an ischemic bout of 12 minutes due to the rarity of the animal surviving a bout longer than 15 minutes, which would not allow for any reperfusion due to a failure to revive the animal (>50% fail to resuscitate) [Kern et al., 1996]. Others have studied the heart in a Langendorff apparatus [van Eyk et al., 1998 , McDonough et al., 1999], but

our study kept the heart *in vivo* during both the ischemic and reperfusion periods until excision of the heart tissue upon conclusion of 12 minutes ischemia with or without reperfusion. This short timeframe of 12 minutes applies more directly for the typical timeframe of approximately 8 minutes for human cases of out-of-hospital sudden cardiac arrest [Cobb et al., 1999]. A longer ischemic bout may lead to the changes other studies have seen, but would likely not result in ROSC and not be relevant to real human emergency cases with such rapid EMT unit response times.

Modifications to the Regulatory Proteins

Degradation of TnI is one of the most common modifications resulting from ischemia/reperfusion especially in localized ischemic episodes, but we did not observe any significant TnI degradation in this study. Proteolytic cleavage of TnI's C-terminus has been seen in many models and longer bouts of ischemia is positively correlated with greater levels of cleavage [Gao et al., 1997 , McDonough et al., 1999 , Foster et al., 2003 , Westfall and Solaro, 1992 , Day et al., 2007 , van Eyk et al., 1998 , Van Eyk and Murphy, 2001]. This TnI truncation alters its ability to interact with TnC and any alteration to this interaction can have drastic impacts on activation of the thin filament, which has been suggested as part of ischemia-induced contractile dysfunction [Gao et al., 1997 , McDonough et al., 1999]. This truncation may alter the muscle's ability to produce force and its Ca^{2+} sensitivity, which can lead to enhanced or even diminished ability to respond to normal Ca^{2+} levels [Westfall and Solaro, 1992 , van Eyk et al., 1998 , van Eyk and Murphy, 2001 , Foster et al., 2003 , Day et al., 2007]. This cleavage, when severe enough, has been shown to influence the position of

Tm on actin such that it increases the probability of myosin binding to actin [Galinska et al., 2010]. SDS-PAGE staining was inconclusive (data not shown) and so mass spectrometry was expected to have a greater resolution for detecting cleavage. Data acquired via LC-MS suggested that there was no difference in TnI truncation between the samples [Fig 11]. This lack of cleavage may also be attributed to the brevity of ischemia in this study, because other studies with 15-60 minute ischemic bouts had increasing amounts of truncation the longer the ischemia occurred [Westfall and Solaro, 1992 , Gao et al., 1995 , Gao et al., 1997 , van Eyk et al., 1998]. In addition, the type of ischemia is important because artery occlusion is a more localized ischemia and removal of the heart into a Langendorff apparatus is a different type of “global” ischemia for the heart, because the whole heart is removed from the animal.

Previous studies suggested other common modifications such as oxidative modifications, but there were no significant differences in oxidative changes to proteins between any of the samples in our study (data not shown). However, we discovered that there was an increase in bis-phosphorylation of Ser23 and 24 in both experimental ischemia TnI samples [Fig 12B] and there was an increase in phosphorylation of α -Tm only in IR2 pig samples [Fig 12A]. Unexpectedly, only the IDNR sample tissue resulted in significantly altered thin filament Ca^{2+} sensitivity and maximal filament velocity [Fig 9 , Fig 10 , Table 1]. The increased maximal sliding velocities and Ca^{2+} sensitivity seen in IDNR samples occurred with only an increase in the bis-phosphorylation of TnI Ser23/24, but this enhanced velocity was lost in the reperfused IR2 sample where there was found to also be an increased level of α -Tm phosphorylation [Fig 12]. Other research supports the possibility that phosphorylation of TnI may allow Tn to be more sensitive to

Ca^{2+} and Tm phosphorylation can change the interaction of Tm monomers and thus activation of the thin filament [Rao et al., 2009]. However, there are conflicting reports of phosphorylation status of regulatory proteins after ischemia as some studies have seen an increase in phosphorylation of TnI and α -Tm [Han and Ogut, 2010 , Han and Ogut, 2011] while other have found no change in PO4 [Rao et al., 2007].

Conclusion

This study was designed to elucidate the underlying structural and functional changes to myocardial contractile proteins caused by global ischemia and reperfusion injury due to sudden cardiac arrest. Our data contradicted the original hypothesis that there would be a decrease of the *in vitro* velocity in samples that underwent ischemia/reperfusion and that this would be due to post-translational modifications to myosin with little or no damage to actin. There was no difference in sliding velocity for across any of the samples when using cardiac myosin with unregulated actin [Fig 6 , Fig 8]. No mass spectrometry was performed because there were no functional effects observed without using the NTF from the samples [Fig 6 , Fig 8]. There may have been alterations to myosin, but in this case none that would be relevant to the scope of this study.

Our original hypothesis predicted that ischemia/reperfusion would affect Ca^{2+} sensitivity of NTF due to cleavage/modifications of Tn. This hypothesis was determined to be partly true, because the IR2 sample was not significantly different from control for either myosin or NTF samples, however the IDNR NTF samples had a significant increase in maximal sliding velocity and increase Ca^{2+} sensitivity [Fig 9 , Fig 10 , Table 1]. Also mass spectrometry was performed on regulatory proteins to quantify the

amount of modifications that occurred to the proteins and the TnI in IDNR and IR2 samples had more bis-phosphorylated TnI compared to control, but only the IR2 sample had an increase in Tm phosphorylation [Fig 12]. Our results agreed with the original hypothesis as there was an increase in Ca^{2+} sensitivity that may have been due to an increase in phosphorylation of TnI, but an unexpected increase in Tm phosphorylation could have mitigated this increase. However, there was no significant cleavage of TnI, although several previous studies have implicated this cleavage as a key player in functional changes due to ischemia/reperfusion [Westfall and Solaro, 1992 , Gao et al., 1997 , van Eyk et al., 1998 , McDonough et al., 1999 , van Eyk and Murphy, 2001]. One study had displayed that phosphorylating TnI with PKA then combining with the rest of the filament proteins into reconstituted thin filament (RTF) increased Ca^{2+} sensitivity and velocity in the *in vitro* motility assay [Hunlich et al., 2005]. This supports that TnI phosphorylation can alter velocity and Ca^{2+} sensitivity such as displayed in our study, however there are inconsistencies across studies of whether TnI phosphorylation increases or decreases velocity [Marston and de Tombe, 2008]. Our findings could suggest that the changes in phosphorylation, increased Ca^{2+} sensitivity, and sliding velocity may be due to a compensatory mechanism in the heart attempting to increase contractility. This phosphorylation mechanism may adversely affect cardiac function depending on the extent of this phosphorylation and which regulatory proteins are affected.

Our findings were different when compared to several other studies likely due to the different techniques, animal models, and timeframes employed for the experimental ischemia/reperfusion. Acquiring more samples from different animals with varying

ischemia/reperfusion times may have allowed us to determine the relation between the duration of the ischemic event with the different modifications seen in other studies.

Another aspect not included in this study was investigating if there were alterations to the thick filament. Studies have shown thick filament alterations can have affects as well, such as phosphorylation of myosin binding protein-C, which is needed for proper cardiac function and if altered could lead to certain cardiomyopathies [Sadayappan et al., 2005]. Different myosin binding protein-C phosphorylation states have been implicated in altering Ca^{2+} sensitivity and maximal sliding velocities [Previs et al., 2012], loaded shortening and power output [Korte et al., 2003], and also can be truncated during ischemia similar to TnI [Sadayappan, 2012]. There can be many things that lead to persistent contractile dysfunction and poor prognosis after ROSC following sudden cardiac arrest, but this study displayed that there were post-translational phosphorylation on muscle regulatory proteins that altered their function *in vitro*.

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